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Inactivation of Cellulase by Shaking and Its Prevention by Surfactants

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The inactivation of *Trichoderma reesei* cellulases by shaking can be prevented by the addition of surfactants and related materials. Highly effective protective agents are fluorocarbon surfactants, and high molecular weight polyethylene glycols. Other compounds, e.g., thymol, have the reverse effect; they *increase* the rate of inactivation caused by shaking.

INTRODUCTION

Denaturation of protein, and the related inactivation of enzymes, by physical means (e.g., shaking and shear) has been reported by Charm (1, 2), Tirrell (3, 4), and Asakura (5). Tanaka (6) and we (7, 8) have found that inactivation of cellulase occurs under the relatively mild shaking conditions employed in the hydrolysis of cellulose. The purpose of this note is to show how this type of inactivation can be minimized.

EXPERIMENTAL PROCEDURE

The cellulase of *Trichoderma reesei* is a complex of *endo*-β-1,4-glucanases (C_x, CMCase)[1] the activity of which is measured by action on carboxymethylcellulose (9), and *exo*-β-1,4-glucanase (cellobiohydrolase, CBH), for which there is no direct measurement in solutions containing C_x. Cellobiohydrolase is required for breakdown of crystalline cellulose; both C_x and CBH are necessary for the rapid hydrolysis of this substrate. In this work, cellulase activity (9) was measured using as substrate crystalline Avicel PH102 (FMC Corp., Marcus Hook, Pa.).

The enzyme preparation employed was a lyophilized culture filtrate of *T. reesei* mutant C30 (10), of the type used for the development of a practical process for cellulose hydrolysis. The effect of shaking was determined by incubating the enzyme (1 mg/ml in 0.025 M citrate buffer, pH 5.0) on a rotary shaker (1-in. circle) at 50°C. Samples were taken at various times, and checked for pH, Avicelase activity, and protein (9).

RESULTS AND DISCUSSION

In the absence of shaking, the cellulases of *T. reesei* are quite stable, even at 50°C (Fig. 1). Inactivation of Avicelase activity increased as the rate of shaking increased. In a prior report (5) it was shown that the deactivation constant increased

[1] Abbreviations used: C_x, *endo*-β-1,4-glucanase; CBH, *exo*-β-1,4-glucanase, cellobiohydrolase.

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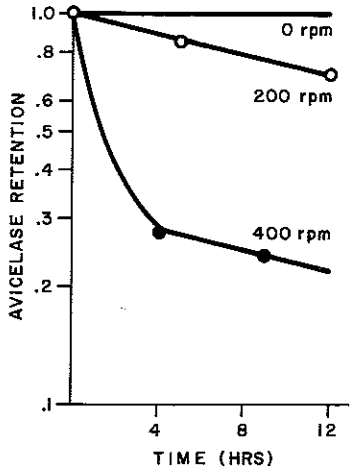

FIG. 1. Effect of rate of shaking on inactivation of *Trichoderma reesei* Avicelase activity. Conditions: 50°C; 1-in.-diameter rotation; pH 5.0.

sharply when the shear stress exceeded 15 dyn/cm^2. The C_x component of *Pellicularia* is similarly inactivated (6).

Proteins have often been used to stabilize enzymes against the adverse effects of pH, temperature and other environmental factors. Few studies have been reported on the protection of enzymes against shear. Metals have been implicated in the shear inactivation of urease (3). We have tested over 50 compounds and find that the inactivation due to shaking can be prevented by the presence of some of them (Fig. 2). Compounds may be highly effective in reducing inactivation (curve A), less active (curve B), or they may *increase* the rate of inactivation (curve C). Proteins (bovine plasma albumin and gelatin) are effective (at 1 mg/ml) but the following substances are protective at much lower concentrations.

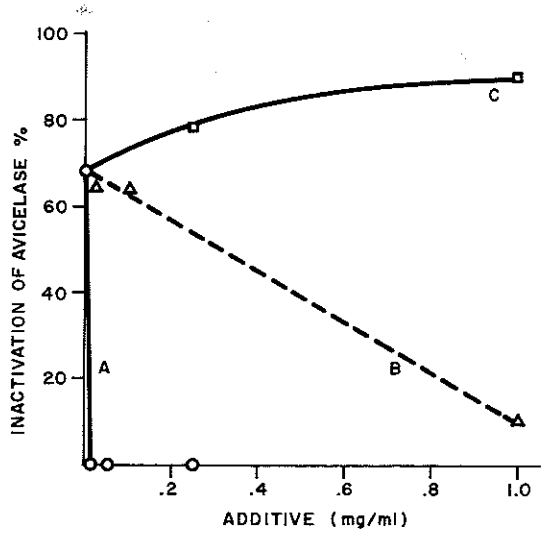

FIG. 2. Effect of various chemicals on inactivation of Avicelase by shaking. Conditions as in Fig. 1; 300 rpm, 1½ h. A, PEG 6000; B, *n*-octanol; C, Thymol.

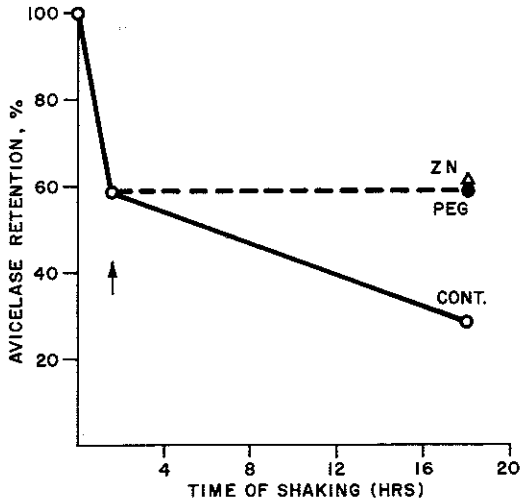

FIG. 3. Effect of addition of protective agents to partly deactivated enzyme. ↑ indicates time (1½ h) at which compounds were added. ZN, Zonyl·N (0.02 mg/ml); PEG, PEG 6000 (0.2 mg/ml); Cont. is a control without any additive. Conditions: 50°C; pH 5.0; 350 rpm.

Effective at 0.001 to 0.01 mg/ml:
Fluorosurfactants: Zonyl FSA, FSB, FSC, FSN (DuPont, Wilmington, Del.)
Effective at 0.01 to 0.1 mg/ml:
Polyethylene glycols 4000–20,000 (but *not* low molecular weight PEG)
Polypropylene glycol; polyvinyl pyrrolidone; Triton X-100; Digitonin; Saponin; methocel; quaternary ammonium compounds.

Rough calculations show that only one molecule of surfactant is required per molecule of enzyme to give maximum protection.

Asakura *et al.* (5) found that various solvents could prevent the denaturation of hemoglobin due to shaking, but the concentrations needed were several orders of magnitude higher than those shown above. Asakura observed that a few solvents, e.g., toluene and chloroform, had the reverse effect, i.e., they actually increased the rate of denaturation due to shaking. In our tests the rate of Avicelase inactivation was similarly increased by the addition of thymol (Fig. 2).

The inactivation due to shaking appears to be irreversible. When added to partly inactivated enzyme, both Zonyl N, and PEG 6000 protected against further inactivation; but neither had any effect on restoration of activity already lost (Fig. 3). The inactivation occurs in the presence of substrate (Avicel) as well as in its absence (7), and the surfactants protect the enzyme under both conditions.

The degreee of protection by surfactants against inactivation by shaking is of a magnitude similar to the effect on heat stability of immobilization of enzymes on various carriers. For example, the half-life of Avicelase under severe conditions of shaking (50°C, pH 5.0, 350 rpm) was found to be 1.5 hours; while in the presence of polypropylene glycol 2700 (0.01 mg/ml), the half-life of the enzyme was 180 h, an increase of two orders of magnitude.

The protective effect of surfactants is not limited to the cellulases of *T. reesei*. Other carbohydrases, conalbumin, and β-lactoglobulin were found to be similarly protected by polyethylene glycol 6000 against denaturation due to shaking.

The inactivation by shaking appears to be the result of both shear and surface denaturation. Under the experimental conditions used protein molecules unfold exposing hydrophobic groups. The subsequent aggregation of protein molecules results from hydrophobic bonding. In the presence of surfactant, the hydrophobic portion of the protein reacts with that of the surfactant, thus minimizing aggregation and subsequent denaturation. The protein–surfactant complex appears to resist conformational changes to a greater degree than does the protein alone.

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